Identification Tool for Hydrocarbon Zones

The present invention is in the field of oil
drilling, exploration and production, in particular
relating to new and improved methods for identifying and
monitoring sources of hydrocarbon (known as hydrocarbon
zones) in the earth's crust (typically confined in
sedimentary rocks). Hydrocarbon (typically petroleum,
i.e. natural gas or oil e.g. crude oil) exploration is
very expensive and many geophysical techniques and
petrophysical techniques are used to produce data which
is used to pinpoint suitable areas for explorative
drilling, and to identify geological formations that may
contain accumulations of petroleum.

Characterisation and identification of oil and
formation water are, in general, carried out by
petrophysical measurements in the well or by geochemical
analysis of fluid samples, but the analytical results
may be difficult to interpret in terms of useful
information regarding important exploration or
production issues, so there is a continued need for
improved or alternative methods of identifying and
characterising hydrocarbons in the geological formations
and reservoirs. The present invention will improve the
interpretation by introducing a new and independent data
set that may also provide information regarding the
migration paths of petroleum from the source rock to the
reservoir(s) and function as an inherent tracer in the
fluids in the production phase.

Exploration wells are drilled in areas of interest
and it is desirable to use these wells to obtain
information about the site, in particular whether
hydrocarbons are present and at what depths. Core
samples obtained from these wells can be analysed for
the presence of hydrocarbons and at present this is

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typically done using petrophysical or geochemical
techniques. However the results of these analyses are
often difficult to interpret so there is a continuing
need for improved or alternative methods of identifying
5   hydrocarbon zones.

In addition, present techniques which might be used
to determine whether a given hydrocarbon sample is from
a certain reservoir are not reliable. Tracers are
difficult to place in different reservoir formations and
10   geochemical techniques based on, for example, a
chromatographic 'fingerprint' of the oils in a sample
may not be specific enough as using this technique it is
difficult to differentiate between similar oils e.g. 2
light paraffinic oils, both rich in n-alkanes.

15   US Patent 5055397 describes a geomicrobiological
exploration method that uses "pathfinder" substances to
identify regions of potential interest for exploration
for oil. The correlation of these "pathfinder"
substances, such as heavy metals, with the presence of
20   oil was used as the basis of this technique. Bacteria
that show resistance to the heavy metals in question are
said to represent genetically encoded tolerance of the
heavy metals and thus their presence is said to indicate
the likelihood of the presence of oil. Tolerance to
25   hydrocarbons is also tested. This technique requires
live samples, although culture is not always required.
The technique is based on the testing of acute toxicity
with a standard set of bacterial inocula, and does not
relate to the testing of indigenous microbes for
30   hydrocarbon source characterisation. The requirement
for live samples means that tests cannot be repeated and
therefore verified subsequent to the testing date.
According to the new methods described herein, live
samples are not required. In addition, the prior art
35   tests rely on substantial amounts of microbes being
present and/or the testing in parallel of samples
against a range of different heavy metals or

hydrocarbons.

In addition, the concentrations of the toxins used
in the assays has to be carefully calibrated as ions,
organic compounds and pH all affects metal toxicity.
5   Whilst this represents one method of identifying
potential oil reservoir sites it can be seen that there
are significant disadvantages in this indirect method of
measuring a phenotype of bacteria, i.e. their ability to
overcome geochemical obstacles that are associated with
10   the presence of petrochemicals, to provide this
information. The presence of bacteria that are tolerant
to heavy metals may not always be a certain indication
of the presence of heavy metals in the area, and
similarly, the presence of heavy metals may not always
15   be strictly correlated with the presence of
hydrocarbons.

A new and improved method to correlate microbial
presence more directly with hydrocarbon zones is
therefore required. Other known methods of
20   geomicrobiological exploration rely on the key
assumption that hydrocarbon reservoirs leak and the
escaped hydrocarbons reach the earth surface, which may
be the sea floor. Thus, the presence of methanogens at
the surface is said to be an indicator of the presence
25   of hydrocarbon zones (Rawat et al. Proc. Second Seminar
on Petroliferous Basins of India Vol. 3 S.K. Biswas et
al. (Eds) 1994 Indian Petroleum Publishers).

This method relies on the ability to correlate the
presence of the methane-utilising bacteria with the
30   remote reservoir. This paper particularly describes
using a gas uptake-manometric technique measuring
utilisation of liquid petroleum gas and propane. The
correlation is only reliable when the geology of the
region is relatively uncomplex as the methane may
35   otherwise migrate significant distances laterally. This
rather crude technique therefore has to be coupled with
geological, geophysical and hydrochemical analysis.

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In addition, this technique relies solely on the
phenotypic traits of the bacteria in question, i.e.
their use of hydrocarbon and this is somewhat
restricting. Thus, even if for example one were to
5   survey for the presence of genes encoding proteins that
relate to hydrocarbon utilisation, this would not
necessarily indicate the presence of hydrocarbons as
genes for hydrocarbon utilisation/degradation are found
widely in nature, even in pristine polar regions and in
10   deep sea trenches. Methane utilising bacteria may
therefore be found in areas that are not associated with
hydrocarbon reservoirs. For example, methanogenic
bacteria may be found in eutrophic lakes, in sea water
and biotopes or natural habitats that are not directly
15   linked to hydrocarbon reservoirs. The methanogenic
bacteria detected and used are normally found at the
cell surface and are not able to live at high
temperatures i.e. they are not thermophiles or
extremophiles.

20   Thus techniques that give a strong and localised
indication of the presence of hydrocarbon zones and also
show good reproducibility and the ability to perform
direct measurement are required.

It is now proposed to exploit the relationship
25   between the presence of certain species of bacteria and
hydrocarbon zones to provide a new way of identifying,
characterising and monitoring hydrocarbon zones.
Certain microorganisms, typically bacteria, live
exclusively or predominantly in or around hydrocarbon
30   zones and may use oil or gas constituents as energy
sources. It is possible to use these bacteria as
markers for the presence of hydrocarbons or to ascertain
certain properties of these hydrocarbons.

The inventors have amassed information from two
35   different reservoirs, and different wells within these
reservoirs to demonstrate the diversity of
microbiological profile of these different samples.

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Whilst bacterial samples have previously been isolated from samples from oil fields, this is the first demonstration of a correlation between a certain microbiological profile and a sample from a particular location.

The bacteria identified in these studies are able to live at high temperatures and are thus called thermophiles or extremophiles. Thermophiles are defined as organisms that can tolerate high temperatures and grow optimally at temperatures above 50°C.

Extremophiles are organisms that grow optimally under extreme conditions, e.g. extremes of temperature, pressure, pH or salinity. The microorganisms which are analysed will preferably be indigenous to the hydrocarbon zone.

The invention therefore relates to a method of detecting, characterising or monitoring a hydrocarbon zone, which method comprises the genotypic analysis of a sample for the presence of one or more (target) thermophilic or extremophilic microorganisms. The hydrocarbon source will typically be in the sub-surface formation and the samples are thus be taken from this formation, or from natural seeps, oil reservoirs or produced oil.

The present invention is of great utility in exploration where detection and characterisation of potential hydrocarbon zones (sources) is of concern. However, it also has application when production has begun, as it enables the produced hydrocarbon and the area in and around the wells to be monitored, i.e. the production conditions to be continually or periodically observed.

In certain embodiments, the thermophilic or extremophilic microorganisms analysed will not be 'target' microorganisms in the sense that their identity is known before the method is begun, rather genotypic information will be gathered and used to characterise

the microorganisms present in the sample. Thus,
alternatively viewed, the present invention provides a
method of detecting, characterising or monitoring a
hydrocarbon source e.g. monitoring the production of
5   hydrocarbons from the various pay zones in a reservoir,
which method comprises the genotypic analysis of
thermophilic or extremophilic microorganisms within a
sample. The nature of the sample is discussed in more
detail below but is typically a sample collected from
10   produced liquids (e.g. oil, water or oil/water mixtures)
from exploration and production wells or drilling out of
a cylindrical core of the reservoir rocks by using a
hollow cylindrical drill pipe (even drill cuttings may
be applicable for the extraction of representative oil
15   and water samples) or the sample may be a sample
collected from hydrocarbon seeps on the sea floor or on
shore; in the upper soil horizon or surface.

"Petroleum" or "hydrocarbon" are used as general
terms for natural gas, condensates, crude oil, heavy
20   crude oil or solid bitumen found in porous rocks. Crude
oil is a complex mixture of naturally occurring
hydrocarbon compounds.

The methods of the invention can be considered
'diagnostic'. In other words, the information obtained
25   through analysis of the microorganisms in the sample
from the exploration/production site is then used in the
further management of that site. The inventors have
shown that the specificity and quality of information
obtained through genotypic analysis of microorganisms
30   present is such as to allow meaningful conclusions about
the hydrocarbon source to be reached. Thus in preferred
embodiments of the methods of the invention, information
regarding the microorganisms present is utilised in an
ongoing exploration or production process.

35   Detection of a hydrocarbon source refers to the
process of identifying the presence of a hydrocarbon
source in the earth's crust i.e. the sub-surface

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formation.   It includes the process of detecting
accumulations of hydrocarbons confined in porous rocks
in the Earth's crust; referred to as petroleum
reservoirs.   The petroleum reservoir should be located
5   in terms of longitude, latitude and depth.   Attempts at
detection are commonly referred to as exploration.

Characterisation of the hydrocarbon zone according
to the invention, e.g. characterisation of the oil in
the reservoir, may involve the correlation of the
10   presence of one or more target microbes with properties
of the hydrocarbons in question.   Characterisation may
be performed as part of a detection method or it may be
performed after the initial identification of a
(putative) hydrocarbon zone has been made.   For example
15   the type of oil, the quantity of oil, the quality of the
oil, the sulphur content of the oil, the presence of gas
or the gas:oil ratio are all factors about which
information is required.   In addition the presence of
asphaltenes, resins and NSO fraction (i.e. nitrogen,
20   sulphur and oxygen bearing compounds) may be determined.
The fact that these properties will affect the bacterial
flora means that it is possible to identify
relationships between the microorganism populations and
these properties, as different microorganisms will
25   thrive under different conditions, e.g. available
sulphur which may be utilised by certain bacteria.   Semi
quantitative analysis using microarrays and quantitative
analysis using diversity indices of fingerprinting
patterns may also be used, in addition to the
30   qualitative analysis.   The depth of a hydrocarbon source
may be identified through analysis of the microorganisms
as different microorganisms live at different depths.

In this way, a pattern or fingerprint of the
hydrocarbon source in terms of its microbiological
35   population may be obtained.   The pattern obtained can be
compared against reference patterns derived from known,
well characterised hydrocarbon zones.   This may relate

to reservoirs, reservoir compartments or reservoir
zones. Thus, a step of comparing the information
obtained regarding the microorganisms present in the
sample against a database of information regarding
microorganisms found in other samples, is a preferred
additional step in the methods of the present invention.
Knowledge about the reference samples can then be used
to infer properties of the new sample.

By "monitoring" it is meant that the production
from a petroleum reservoir or reservoir zone is being
observed as a function of time. Produced oil (or water)
samples may be analysed at regular intervals as a part
of the monitoring process, and changes in its microbial
population may be detected. These microbial changes may
be used to infer the contribution to the oil production
from the various reservoir zones in a commingled
production scheme (provided that their inherent
microbial populations are different) and also to infer
the approach of injection water from the injector
towards the production well. In this way, the dynamic
reservoir behaviour can be monitored without complex
geochemical analysis or by using artificial tracer
compounds that has to be injected into the reservoir and
produced back with the oil or water.

Thermophilic and extremophilic microbes are also
associated with a number of oil reservoir problems, like
corrosion, near-well plugging and reservoir souring.
Many of these problems are related to the activities of
sulphur-utilising microorganisms. The high content of
small organic acids in many reservoirs combined with
anoxic conditions, as well as concentrations of sulphate
and hydrogen are important for the activities of
strictly anaerobic sulphidogenic and methanogenic
microbes. Microbial consortia have been found in
reservoirs not associated with any microbial problems,
without any injection water penetration, and with
temperatures >80°C. This indicates that many hot oil

reservoirs harbour an indigenous flora which is not
introduced by drilling muds or injection water and which
may be stimulated during specific reservoir conditions.
Changes in this flora during production may be observed
5   in order to avoid enrichment of unwanted growth with
subsequent reservoir problems as described above. Such
techniques may also be considered "monitoring".

In addition, the high sensitivity of bacterial
populations to changes in their growth conditions may
10 provide an early warning system for changes that are yet
to take place such as biofouling (e.g. H_2S production or
plugging). For example Archaeglobus is present in sour
reservoirs (i.e. those that contain free sulphur) and
may thus be used as an early warning system for the
15 presence of H_2S production. Arcobacter also may appear
in hot oil reservoirs. These oxidise reduced sulphur
compounds (e.g. H_2S) and their presence will also
therefore act to indicate the likelihood of reservoir
souring. Other bacteria are associated with other
20 changes in the chemical condition of the reservoir. In
this case, the reservoir from which the sample is taken
may be the producing reservoir and the sample may be oil
or hydrocarbon directly taken from the reservoir or well
water etc.

25   Thus information on the behaviour of the
hydrocarbon zone (e.g. fluid saturation) as a function
of time can also be derived from the generation of data
concerning the particular species or types of species
that are present in the sample.

30   Prior art studies of hot reservoir microbes are
based on culturing in specific synthetic enrichment
media with subsequent isolation and characterisation of
the growing organisms. However most of the
extremophiles living in such areas are difficult to
35 culture. Therefore the present invention, which
provides a culture independent method represents a
significant advance over the prior art.

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For methods of correlating the presence of microbes with changes in oil reservoir properties, it may be desirable to have just one or two species of target microorganism, e.g. Archaeglobus.

5   H₂S production for example can be indicated by the presence of sulphur reducing bacteria (SRB). This is problematic for oil production as SRB can cause reservoir souring via the metabolism of sulphate. Reservoir souring and H₂S production is a problem for oil
10   generation in that H₂S has to be removed from the production streams. In addition, hydrocarbon with a high sulphide content is less valuable than pure hydrocarbon. Deposition of iron sulphide deposits in the reservoir is also a feature of reservoir souring
15   (biofouling). Thus, a means of detecting SRB in producing reservoirs would give a good early warning indication of the production of H₂S and enable early intervention to take place.

In addition to the production of H₂S, the
20   microbiological profile of production reservoirs can be used to survey for the presence of bacteria known to generate high biomass with plugging potential. When plugging appears, considerable numbers of sulphate reducing microorganisms are seen. Other microorganisms
25   may also be responsible. For example, in hot oil reservoirs, Archaeoglobus, Acinetobacter, Desulfotomaculum, Methanobacterium, Methanococcus, Methanoculleus, Nanobacterium, Petrotoga, Pseudomonas, Geothermobacter, Ralstonia, Sphingomonas, Spirochaeta,
30   Thermoanaerobacter, Thermococcus, Thermotogales, Thermodesulfobacterium, Thermodesulforabdus, Thermodesulforamonas, Thermotoga were identified in addition to other microorganisms phylogenetically related to Erythrobacter, Arcobacter, Aquabacterium,
35   Peptostreptococcus, Pseudomonoas and Archaeoglobus. Again early intervention will prevent expensive clean-up operations.

As well as providing an early warning of potential
problems with a reservoir, the monitoring methods of the
invention can be used to evaluate the progress of
techniques used to overcome previously identified
5   problems.

Any sample (e.g. from the hydrocarbon zone itself
which may include a seep zone that is capable of
supporting a microbial population may be used in the
invention. In particular, the sample can be defined as
10   any naturally occurring sample (e.g. earth, dust, shale
or oil) containing liquid hydrocarbon and/or water that
has previously been exposed to hydrocarbon. The
composition of the microbial population is typically
sensitive to either the presence of hydrocarbons or
15   changes in hydrocarbon properties. The microbial
population of the sample may only be indirectly
sensitive to the presence of hydrocarbons. For example,
analysis of samples from known oil fields indicate that
rock near a hydrocarbon source or even rock which is
20   often but not inevitably associated with a hydrocarbon
source will contain certain populations of
microorganisms. The sample will preferably be a core
sample obtained from an exploration well or well water.
The core sample may be analysed at different points,
25   i.e. relating to different depths in the crust. In
order to perform the analysis, the cores may be crushed
or cut and then shaken with buffers to enable the
isolation of the microbial material for analysis. The
microbial material may for example be the microorganisms
30   themselves but is preferably the nucleic acid extracted
therefrom. The samples may conveniently be filtered
through membrane filters, trapping microorganisms on the
filter surface. The buffer used in this stage will
depend on the nature of the further analysis of the
35   sample and the nature of the sample. Different
extraction methods which may be used are described in
the Examples.

The sample may not be a surface sample e.g. not a
sample collected from the surface or upper 2 metres of
body of soil or sediment. The surface being in direct
contact with air or with water. The sample is
5   preferably collected from a core penetrating the
potential hydrocarbon-bearing formations.

Particularly in the circumstances where production
has begun, the sample may consist of or comprise
hydrocarbons e.g. it may be an oil sample. Its analysis
10   will allow characterisation of the hydrocarbon itself.

The exploration for petroleum (typically oil and
gas confined in sedimentary rocks in the earth's crust)
is very expensive, and many types of data (mainly
geophysical and petrophysical data) are used to identify
15   geological formations that may contain accumulations of
petroleum.

Alternatively, the sample may be sediment from the
sea floor seep zone or, for the purposes of
characterising or monitoring, the sample may be an oil
20   or petroleum sample. If oil is the sample, then the
microorganisms and nucleic acids are separated from the
oil using a detergent such as sodium dodecyl sulphate,
washing the oil with a water based buffer or by phenol
chloroform isoamyl extraction. This is usually combined
25   with mechanical disruption generated by stirring in a
suspension of small plastic beads, also possibly in the
presence of nucleic acid extraction chemicals such as
phenol-chloroform. Such techniques are well known in
the art. Commercial kits are also available for nucleic
30   acid extraction. If the sample is water, it is filtered
through 0.22 μm filters prior to treatment. The sample
may also be a mixture of oil and water or any other
substance that is extracted from the production well.
It may also be formation (reservoir) water, water or
35   sediment from seep zones.

The analysis of the sample may be performed in a
number of ways. It is important that the analysis is

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genotypic rather than phenotypic as this allows for a
more accurate picture of the microbial population in the
sample to be achieved. Methods that are independent of
any culturing of the microorganisms in the sample are
5   particularly preferred. A further advantage of the
present method is that the samples do not even need to
contain live microorganisms for the analysis to be
performed. The specificity of nucleic acid/nucleic acid
or protein/nucleic acid interactions means that reliable
10   results can be achieved on only a small sample without
the need for culturing. Also, there are regions within
the bacterial genome which are highly conserved between
bacterial species and other regions which are species
specific. Therefore regions may be targeted to give
15   information about the total bacterial population, about
classes of related bacteria or about individual species.
        The present invention preferably provides
information regarding the microbiological profile of the
sample. The 'microbiological profile' will vary
20   depending on the circumstances and it may be that
determining the 'microbiological profile' will only
comprise confirming the presence or absence of a single
target microorganism or class of microorganisms which is
considered indicative of a certain grade of hydrocarbon
25   or, and this may be more appropriate during production
rather than exploration, of a problem with the well such
as proliferation of H_2S producing microorganisms.
        However, preferably, the microbiological profile
will comprise qualitative (and possibly quantitative)
30   information about more than one species or class of
microorganism. This will mean a series of separate but
possibly simultaneous tests will be performed on the
sample, each one designed to target a certain species or
class of microorganisms. The word 'class' is used
35   herein in a very general sense to mean a group of
microorganisms which are related functionally or
taxonomically. As the analysis performed is genotypic,

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the groupings will typically be through sequence
similarities and conserved regions, for example a probe
or primer may target a range of bacteria through
hybridisation to a sequence which is conserved (or
5   substantially conserved) between them. The number of
'tests' performed will typically be between 2 and 20,
preferably 3 or 4-8.

It is not necessary to identify every species that
is contained in the sample, as it is often possible to
10 correlate the property that is being assayed for or
obtain reservoir distinguishing information by the
presence of one or a few species. Usually between 2 and
10 tests will be performed, preferably 3 or 4-8 if the
samples are being analysed with different probes
15 simultaneously. The sample may be analysed for the
presence of each species sequentially or simultaneously,
depending on the technique used. If the samples are
analysed sequentially there is no upper limit on the
number of species that can be assayed for. In a
20 preferred embodiment, the microbiological profile of the
sample is determined in a single test.

'Target microorganisms' may include microorganisms
that metabolise hydrocarbons or use them for growth,
however it is preferable that the target microorganism
25 is not identified based on the presence of genes that
encode proteins that are involved in hydrocarbon
metabolism or by the phenotypic ability of the
microorganism to metabolize hydrocarbons. These genes
encoding enzymes involved in hydrocarbon metabolism are
30 widespread in nature and are not useful as accurate
markers for the presence of hydrocarbon zones. Target
microorganisms in general may include different types of
bacteria (including cyanobacteria), other prokaryotes
and Archaea including anaerobic methaneogens, anaerobic
35 and aerobic sulphur-metabolizing bacteria, halophilic
Archaea and moderately thermophilic Thermoplasmales.
Preferred target microorganisms are Archaea,

sulphur compound utilising microorganisms, fermentative
bacteria, manganese and iron reducers, methanogens and
acetogens. Specific microbes which may be target
microorganisms are mentioned herein.

5   The methods of the invention involve 'genotypic
analysis' as opposed to phenotypic analysis, i.e.
investigations of the genetic constitution of the
microorganisms through direct analysis of nucleic acid
rather than the physical expression of the genetic

10   information. RNA but more preferably DNA is analysed.

In a preferred embodiment of the invention, nucleic
acid based amplification methods such as PCR are used to
identify and characterise the microorganisms. The use
of species specific sequences of nucleic acid provides a

15   very accurate method of microorganism typing. For
example the bacterial genome has regions which show very
little variation from one species to another and
different regions which are highly variable and
essentially species or even sub-type specific. Regions

20   of the genome can be targeted for identification
purposes which are more or less variable depending on
whether it is desired to use a single microorganism
species or a group of microorganisms as a marker for the
presence of hydrocarbons. Similarly regions of the

25   genome that are identical in a group of microorganisms
can be used to identify that group.

Nucleic acid techniques such as PCR also display
the advantage that they do not have to be performed
immediately following the isolation of the sample, in

30   contrast to techniques that rely on regrowth or testing
of bacteria isolated from samples. Cultivation of
bacteria is known to provide a very biased view of
microbial diversity, as it is difficult to correctly
predict growth conditions. It has been estimated that

35   less than 1% of all bacteria have been identified from
culture techniques. Particular species will not be
identified if the culture conditions used are not

optimal for their growth. For example when culture and
nucleic acid techniques were compared for identification
of microorganisms from hot oil reservoirs, methods that
rely on the isolation and regrowth of bacterial samples
5   identified Archaeoglobus, Acinetobacter,
Desulfotomaculum, Methanobacterium, Methanococcus,
Methanoculleus, Nanobacterium, Petrotoga, Pseudomonas,
Ralstonia, Sphingomonas, Spirochaeta,
Thermoanaerobacter, Thermococcus,
10 Thermodesulfobacterium, Thermodesulforabdus and
Thermotoga, whereas culture-independent methods led to
the identification of other dominant microbial species,
phylogenetically related to Erythrobacter, Arcobacter,
Aquabacterium, Peptostreptococcus, Pseudomonoas and
15 Archaeoglobus. Culture based techniques are also time
consuming and may take several days or weeks to perform.
They are also not suitable for on-site analysis given
the requirement for specialised culture equipment. In
some instances the most comprehensive picture of
20 microbial flora may be obtained through a combination of
culture-independent and culture-dependent techniques.

When characterisation is carried out via genotyping
performed using PCR or other nucleic acid based
techniques it is possible to store the samples using
25 methods that kill the microbes yet do not destroy their
DNA. Examples of such methods include freezing and
fixation in solvents such as ethanol, toluene,
formaldehyde containing buffers or sucrose. Thus
samples containing live or dead microbes are equally
30 suitable for analysis using nucleic acid methods, in
contrast to culture based techniques and the technique
of US 5055397.

Samples may therefore be transported or stored
without losing their utility. This also allows repeated
35 analysis of the samples to be performed, after periods
of storage, for example to confirm results or to screen
for microbes which may be have been identified

subsequent to the isolation of the sample. In this way
a more complete profile may be built up, thus increasing
the accuracy of the predictions derived from this
technique.

5   The sample may contain only a small number of
individual microorganisms and the amount of time
required to generate sufficient numbers to perform
standard microbiological analysis may be considerable.
In contrast, nucleic acid techniques such as PCR require
10   only a small number of cells, and indeed in some
instances results can successfully be achieved by
performing PCR on samples containing single cells.

          rDNA or ribosomal DNA is present in all living
organisms and encodes ribosomal RNA (rRNA), the subunits
15   associated with the ribosome. There are 3 types of rRNA
in microbes, 5S, 16S and 23S. The sequence of these
molecules and hence the DNA encoding them, is highly
conserved throughout evolution as ribosomal RNA has the
same function in cells of all life-forms. There are
20   also regions of significant variability. These
sequences can therefore be used to determine the
evolutionary relationship between two organisms, a
process known as phylogenetic analysis. The presence of
a target sequence of 16S rDNA specific to a certain
25   species may be detected by the presence of a delivered
PCR product, which is not seen in other species.

          Species specific differences in the sequence of 16s
rDNA can therefore be utilised as markers for the
identification of microorganisms. Probes that bind
30   specifically to these target sequences may be used as
PCR primers. Alternatively the probes may be used to
bind to and thus specifically identify PCR products or
to detect target DNA sequences directly (e.g. using FISH
or microcyte® or on DNA chips). Thus in a preferred
35   embodiment of the invention 16S rDNA sequences are used
as target sequences for the identification of
microorganisms.

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In a preferred embodiment, the probes are not labelled but are used to perform an amplification reaction, e.g. through use of the polymerase chain reaction (PCR). Thus these probes may hybridise to target regions within the bacterial genome which are specific to a particular species or class of bacteria and result in amplification of the DNA only from target species. Alternatively the primers are designed to surround the target sequence and thus amplify a region of interest for further analysis. Performance of amplification reactions and detection of amplicons are standard techniques.

Detection through the use of amplification reactions where the probes can act as primers for an extension reaction only when hybridised to target regions is very sensitive; only a small number of bacteria, theoretically only one bacterium, is sufficient to generate a detectable signal if enough rounds of the amplification are performed. The specificity of the system can be varied due to the stringency of the probe hybridisation conditions employed.

Thus, the target DNA sequence may be the site of primer binding and therefore the probe may be a PCR primer. In the presence of the specific sequence in question, a PCR product of the desired size will be generated and can be detected using standard methods such as gel electrophoresis. This may be followed by Southern blotting, or other detection techniques in which case a further probe may be used to detect any characteristic or specific sequences that are contained in the resultant PCR product.

Probes may be designed that are not specific to a single species. It is therefore possible to detect more than one type of microbe with a single pair of general primers or using a single probe to identify sequences that hybridise to that sequence e.g. in FISH, on a

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microchip or using a conventional blotting technique.
For example, primers referred to herein as f21ARCH and
r958ARCH and f341Bac/r907Bac have been used to identify
bacteria and Archaea respectively. Amplification
5   products generated using these relatively non-specific
primers may then be further analysed to yield more
specific information about the microorganism populations
using RFLP, DGGE, Southern blotting etc. as described
herein.
10   By sequence specific it is meant that under the
conditions used in the assay, the probe or primer only
binds to the target DNA sequence and to no other DNA
sequence.
            The target DNA sequence is the DNA sequence that is
15   used to characterise the particular microbe and may be
unique to the species to be identified or common to a
group or class of species to be identified. The
sequence specific probe may recognise the target DNA
sequence directly or could be used to amplify a region
20   containing the target DNA, the product of which is then
subjected to further analysis e.g. using hybridisation
techniques, to characterise the specific target
nucleotide sequence.
            Typically the target sequence is not part of or
25   associated with a gene whose product is involved in the
metabolism of hydrocarbons.
            If the sequence specific probe is a primer that
recognises the target DNA sequence then the presence of
an amplified product of the appropriate size, using
30   standard gel electrophoresis will indicate that the
microbe containing the target sequence in its nucleic
acid (e.g. its rDNA) is present in the sample. This may
optionally be further confirmed by subjecting the
amplified product to hybridisation techniques such as
35   Southern Blotting. These techniques are well known in
the art. If the sequence specific probe is designed
such that the region of DNA that may contain the target

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sequence is amplified, then the presence of an amplified
product is not sufficient to identify the presence of
the target microbe. Further analysis of the amplified
product is required to determine whether the target
5   sequence is indeed present. This may be performed using
Southern Blotting with sequence specific probes that
have been labelled e.g. with radioactivity. These
probes will bind specifically to target DNA sequences
and not to other DNA sequences. A positive
10   identification of the target sequence would therefore
result from the amplification of a region of 16s rDNA
that contained the correct target sequence.

        Such probes may also be used directly on the DNA
sample, without amplification first.

15   Denaturing gradient gel electrophoresis (DGGE) may
be also used to analyse the PCR products. This
technique is used to separate nucleic acid molecules
which exhibit different melting conditions, due to
variations in their sequences. The fragments, in this
20   case the PCR products, are run on a low to high
denaturant gradient acrylamide gel in which they are
initially separated based on molecular weight. In the
higher denaturing conditions, sequence specific DNA
melting starts to occur which affects the mobility of
25   the fragments. The mobility shift differs for different
sequences and as little as 1 bp difference can cause a
mobility shift. In this way species specific
differences in amplified fragments of 16s rDNA can be
detected in target microorganisms.

30   The PCR products may also be analysed using
restriction fragment length polymorphisms (RFLPs).
These are changes in DNA sequence that lead to a change
in one or more restriction enzyme sites. So if a
species specific change in a 16S rDNA sequence causes
35   the appearance or disappearance of a restriction enzyme
site then by digesting the amplified PCR product with
this enzyme, followed by standard gel electrophoresis

analysis to ascertain the molecular weights of the products of this digestion, the target species may be identified. If RFLP analysis is to be used then the PCR primers are designed to amplify a target DNA region containing an RFLP site. Unique RFLPs may be used to identify particular microbial species.

Any other standard technique known in the art to detect specific known nucleotide sequences in PCR products may alternatively be used. The amplified products may be sequenced directly and, as described in the present Examples, the resulting sequence information subjected to BLAST analysis.

It is preferred that the analysis is carried out using multiple probes or primers sequentially or simultaneously, in order to generate information regarding the microbiological profile of the sample. In a most preferred embodiment the analysis is performed simultaneously using multiple probes or primers, i.e. a battery of probes or primers. The battery of probes or primers will be specifically designed depending on the nature of the information that is required, i.e. the range and types of microorganisms which it is desired to detect.

Other nucleic acid based techniques, in addition to PCR or instead of, may also be used to identify bacteria present in the sample. Such techniques include those where a probe binds directly to the nucleic acid of the microbe without prior amplification of the target sequence. In this case the probes that bind to the target nucleic acid sequence are labelled to enable their detection following hybridisation. Accordingly, a further aspect of the invention relates to methods of identifying a hydrocarbon source through analysis of a sample for the presence of one or more, specific nucleic acid target sequences or markers wherein the analysis is performed by FISH, microcyte® or using a DNA microchip.

FISH (fluorescent in situ hybridisation) is a well

characterised technique that is used to detect target
nucleic acid sequences in individual intact cells via a
system of coupled antibodies and fluorochromes.
Specific nucleic acid probes of approximately 20
nucleotides in length are synthesised, incorporating
labelled nucleotides. The nucleic acids may be labelled
by the conjugation or addition of fluorescent molecules
such as FITC, Cy5, Cy3, TRITC, Texas Red. Alternatively
the probes may be labelled with an immunogenic molecule
such as digoxygenin, in which case antibodies to the
immunogenic molecule are used in the detection step.
The antibodies might be fluorescently labelled or may be
detected by other fluorescently labelled antibodies.
Biotin may also be used to label the probes, and this is
detected using its high affinity binding partner avidin.
The probes may be targeted to DNA or preferably to rRNA.

The hybridisation is carried out by mixing the
labelled probes with the sample, in which the nucleic
acid has been denatured, thus providing access of the
probe to the target DNA.

The probes are detected directly, using
fluorescence microscopy or indirectly, following the
addition of fluorescently labelled antibodies that
specifically bind to the labelled probe, or to other,
non-labelled antibodies that specifically bind to the
labelled probe. It is possible to detect more than one
type of fluorescence simultaneously, using the
appropriate filters. In this way, the detection of the
presence of more than one marker can be performed
simultaneously.

If FISH is used, the probes will be designed to
specifically hybridise with the identified target
sequences for each microbial species or group of
species.

FISH may be combined with other stains such as
Ethidium Bromide and DAPI. This would allow the
calculation of the number of microorganisms containing

the target sequence relative to the total number of microorganisms present.

Microcyte® flow cytometry can also be used to detect or analyse the presence of specific bacterial species group of species. Microcyte® flow cytometry is a flow cytometry technique that is specifically adapted for microbiology and is advantageous in that it can be used with very low cell numbers. The cells are stained using fluorescently labelled rRNA probes. In this way, cells containing specific sequences can be identified and quantified.

Applying probes to a microchip and allowing sample nucleic acid to hybridise is a further way of identifying sequences homologous to (or sufficiently homologous to bind to) these probes. The probes to which sample DNA hybridises can be readily identified if the sample nucleic acid has been labelled. The probes are attached to the chip at known locations and the location of the signal indicates to which probe the nucleic acid has bound. The sample nucleic acid for example may for example be genomic DNA, total RNA, mRNA, cRNA, cDNA, PCR product and each of these or any other nucleic acid may be labelled prior to contacting with the chip according to standard techniques.

From all the above discussions, it is clear that the methods of the invention typically comprise a step wherein an oligonucleotide binds to a region of nucleic acid within the nucleic acid of or derived from the target microorganisms. Thus the "genotypic analysis" preferably comprises contacting the sample with one or more species of oligonucleotide, said oligonucleotides being designed to hybridise with or adjacent to characterising sequences within the nucleic acid of target microorganisms. The term oligonucleotide encompasses probes (e.g. labelled probes) and primers for amplification or sequencing-by-synthesis methods. Unless otherwise clear from the context, the terms

probes and primers may be used interchangeably herein.
The 'characterising sequences' are those target
sequences described above which may be species or class
specific. As previously mentioned, where an
5   amplification reaction is performed, primers may
hybridise adjacent to (i.e. one on each side of) a
characterising sequence. This also presents the
opportunity for two layers of specificity if selective
primers are chosen and then a further hybridisation
10   reaction to analyse the amplified sequence is also
performed.

As preferred variants of all of the above methods,
a number of different probes or primers which hybridise
to target sequences within different bacterial species
15   may be used simultaneously, which will give a pattern of
the bacterial flora present in a sample i.e. a
microbiological profile of the sample. The pattern may
be visualised following gel electrophoresis in the case
that it is produced by PCR. Alternatively, as mentioned
20   above, FISH, microcyte or other nucleic acid based
technologies that permit the visualisation of multiple
probes in a single sample may be used. Such a system
which provides more detailed information about the
bacterial species present may give a good indication of
25   the conditions in the environment from which the sample
was taken. The pattern obtained can be compared against
reference patterns derived from samples from known
hydrocarbon zones.

The analysis (preferably the simultaneous analysis)
30   of multiple species to generate a microbiological
profile therefore represents a preferred embodiment of
the invention.

In preferred embodiments, the methods of the
present invention (more particularly the exploratitive
35   methods of the invention) result in the generation of a
microbiological profile for a given sample. Qualitative
information about the presence or absence of 2 or more

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types (i.e. species or groups/classes), preferably 3 or
4 or more, e.g. 3 or 4-8 different types. This results
in the generation of a pattern which can be compared,
manually or through computer analysis with reference
5   samples to provide a good indication as to the
hydrocarbon potential of the sample.

Thus, in a preferred embodiment the present
invention provides a method of evaluating a sample (e.g.
from a sub-surface formation, a seep sample or an oil
10   reservoir sample) by the genotypic detection of a
plurality of different target thermophilic or
extremophilic microorganisms, the generation of a
microbiological profile for said sample and optionally
comparison of said profile with one or more reference
15   profiles. The evaluation may be to determine the
hydrocarbon potential of, or the type of oil that may be
present in, the area or region from which the sample was
taken or it may be part of an analysis or monitoring
programme performed on a known reservoir or well in a
20   reservoir e.g. a production phase sample. The term
'sub-surface formation' is the term generally used for
the region of the earth's crust in which hydrocarbons
are located.

The evaluation may alternatively provide
25   information with respect to migration routes, reservoir
fill/spill evaluations and reservoir depths.

A further aspect of the invention relates to the
surveillance and analysis of oil from different
formations in a commingled production in the production
30   phase, i.e. where the produced oil is derived from two
or more reservoirs. This will occur if there is
reservoir continuity. Alternatively, commingling of oil
from different reservoirs may also occur if there are
fault lines. The technique may also be used to detect
35   whether two oil samples drawn from different wells are
in fact derived from a single continuous reservoir or
from separate reservoirs. In the production phase, the

evaluation can be used to identify the producing zones
in a commingled production scheme and monitoring the
approach of injection water towards the production well.
It is critical to oil field management that the

5   structure of the reservoirs should be understood.
It will be appreciated that once the individual
microorganism profiles been established for the two or
more reservoirs or producing zones, it will be
straightforward to determine during production if the

10   oil product is in fact a mixed product from the two
reservoirs by analysing its microorganism content. For
example if species A, B and D are found in one reservoir
or producing zone, and species B, C and E are found in a
second producing zone, then the presence of only species

15   A, B and D indicates that the oil is coming from the
first reservoir or producing zone, and action can be
taken to shut off or modify production from the second
(B, C and E containing) zone. Quantitative analysis may
indicate the relative contributions of one or more

20   producing zones to a collected hydrocarbon.
In the art commingling has been investigated by
pressure testing and mapping or by injecting tracers
such as radioactive tracers or organic fluorescent
substances into the different reservoirs. Suitable

25   tracers may be pressure testing is very expensive and
also not always possible. Injection of tracers into the
reservoir formation is a very difficult procedure and
also not always possible. It may be possible to
evaluate natural existing components in the oil or water

30   which may be specific for a particular reservoir in
order to determine the source of commingled products,
however such reservoir specific components may not
always exist.
A method that can be used for any oil field is

35   therefore required which does not require production to
be ceased and which can be used for continuous
monitoring.

By analysing the microbiological profile of the produced oil or water it will be possible to correlate either the presence of certain microbes, or the overall pattern of microbes with the source of the oil.

Analogously, in the exploration phase, migration routes can be studied, by comparing the microbiological profiles of samples collected at different locations.

Reservoir fill/spill evaluations may also be performed in this way. If for example oil from one reservoir has spilt over into a second reservoir then the presence of new microbiological species in the other reservoir may indicate the origin of the spilt oil.

Microorganisms that may be identified and whose presence is correlated with the characteristics and presence of hydrocarbons include representatives of bacteria and Archaea. Archaeoglobus, Acinetobacter, Bajacaliformiensis, Desulfotomaculum, Methanobacterium, Methanococcus, Methanoculleus, Nanobacterium, Petrotoga, Pseudomonas, Ralstonia, Sphingomonas, Spirochaeta, Thermoanaerobacter, Thermococcus, Thermodesulfobacterium, Thermodesulforabdus, Thermotoga, Acidoaminococcus, Aminobacterium, Halomonas, Desulfomicrobium and Methylobacterium, have all been identified in oil fields and may therefore constitute target microorganisms.

The ability of many Archaea to survive in the conditions found in production and explorative wells make them particularly suitable as target microorganisms. Generally, Archaea can be identified by their cell wall which lacks a peptidoglycan skeleton and by their cytoplasmic membrane which contains glycerol ethers with C_{20} (phytanyl) and C_{40} (biphytanyl) alkyl isoprenoids in place of the fatty acid glycerol esters. In addition, the DNA-dependent RNA polymerases of Archaea differ from those of Bacteria in that they consist of more than four subunits and are resistant to the antibiotics rifampicin and streptolydigin.

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These and other microbes are specially adapted to
live under the conditions found in oil wells. For
example they are thermophiles or extremophiles. They
may also be methanogenic, sulphur using, or able to live
5   at great depths.

A further use of the evaluation and
characterisation techniques described herein is to
evaluate reservoir depths. There is a direct
correlation between temperature and depth. There is
10   also a direct correlation between temperature and the
ability of microorganisms to grow and therefore the
presence of certain microorganisms provides information
directly as to the temperature and indirectly as to the
depth of the sample.

15   As it is not necessary that the sample contains
live microorganisms, it is also possible to derive
information concerning past properties of the oil, as
long as the nucleic acid of the microorganism(s) that
provide this information is preserved in the sample.

20   As the properties of oil may be changed by the
presence of one or more microorganisms, through the
alteration and/or metabolism of components of the oil,
(oil degradation) it is useful to know whether the
current properties of an oil sample are inherent
25   properties or whether they have been brought about by
oil degradation. The presence of certain microorganisms
known to be associated with biodegradation can be used
either to diagnose biodegradation and could allow early
intervention with suitable biocides.

30   The invention also relates to probes or primers for
use in the method of analysis. For example PCR primers
that recognise species specific 16s rDNA sequences and
thus generate species or class specific amplification
products form part of the invention. As mentioned
35   above, the PCR primers may also be designed to
specifically amplify DNA sequences containing the target
regions of 16s rDNA that contains a species specific

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sequence that can then be identified using RFLP analysis
or hybridisation analysis techniques that are known in
the art.

The probes may alternatively be used to bind
directly to nucleic acid prepared from or present in the
sample without prior amplification.

Various new probes are described herein which are
suitable for identifying individual species or groups of
species. Such probes themselves constitute a further
aspect of the present invention.

The probes or primers may recognise a single
species or a class of related species. For example
there are primers/probes that will identify bacteria;
f341Bac and 907Bac, 341fBac: 5'-CCT-ACG-GGA-GGC-AGC-AG-
3' (SEQ ID NO: 1) (forward primer) 907rBac: 5'-CCC-CGT-
CAA-TTC-CTT-TGA-GTT-3' (SEQ ID NO:2) (reverse primer),
which give an expected PCR product of 567 bp. There are
also primers/probes that identify Archaea, f21ARCH and
r958ARCH 21fARCH:
5'-TTC-CGG-TTG-ATC-CCG-CCG-GA-3' (SEQ ID NO:3) (forward
primer) 958rARCH: 5'-CCC-GGC-GTT-GAA-TTC-AAT-T-3' (SEQ
ID NO: 4) (reverse primer) which give an expected PCR
product of 938bp (Teske et al. 1996 Appl. Environ.
Microbiol. 62: 1405-1415, DeLong et al. 1992, PNAS USA
89(1): 5685-9). There are also primers that recognise
groups of sulphur reducing bacteria (Desulphovibrio)
e.g. f341Bac and r687SRB (r687SRB: TACGGATTCACTCCT) (SEQ
ID NO: 5). Probes/primers that recognise Desulfovibrio
and Desulfobulbus (f385SRB:
5'-CGG-CGT-CGC-TGC-GTC-AGG-3' (SEQ ID NO: 6) and
r907BAC) and probes/primers that recognise Desulfobacter
and Desulfobacterium (f341Bac and r804SRB:
5'-CAA-CGT-TTA-CTG-CGT-GGA-3') (SEQ ID NO: 7). These
may be used for PCR or for direct hybridsation
techniques.

Probes that are suitable for use in FISH, for
application to a microchip or for other hybridisation

techniques include EUB338: 5'-GCTGCCTCCCGTAGGAGT-3' (SEQ
ID NO: 8) (against bacteria generally); ARCH915: 5'-
GTGCTCCCCCGCCAATTCCT-3' (SEQ ID NO: 9) (against all
Archaea generally); NON338: 5'-ACTCCTACGGGAGGCAGC-3'.
(SEQ ID NO: 10) (negative to all bacteria and Archaea);
ARGLO605:
5'-GCCTCTCCCGGTCCCTAG-3' (SEQ ID NO: 11) (against all
registered Archaeoglobus); THERSI672: 5'-
CCCTACACCAGCAGTTCC-3' (SEQ ID NO: 12) (against all
registered Thermotogales). Ery368:
5'-CCAGTATTCTAGCCATCC-3' (SEQ ID NO: 13) (for all
registred Erythrobacter and Erythromicrobium); ARC293:
5'-TCCATCTACCTCTCCCAY(C or T)-3' (SEQ ID NO: 14) (for all
registered Arcobacter).

        GCGGCTWCCTGGACCACCGATACT, (Desulforomonas;
Geothermobacter SEQ ID NO: 15), GGCGGTGAAATTTTGCAGCTCA
(Baceroides, SEQ ID NO: 16) and GTAGGCGGAATTTGTGGTGTAGC
(SEQ ID NO: 17) are three further probes that may be
used.

        554 ARCHI 5'-TTA-GGC-CCA-ATA-ATC-MTC-CT-3', (SEQ ID
NO: 18) is a probe that has been used for Southern
Blotting and may be used in any direct hybridisation
technique to recognise Crenarchaeota (group I Archaea).
544 ARCHII 5'-TTA-GGC-CCA-ATA-AAA-KCG-AC-3', (SEQ ID NO:
19) is a further probe that has been used for Southern
Blotting and may be used in any direct hybridisation
technique to recognise Euryarchaeota (Group II Archaea).
        Thus the novel probes described above or their
complements represent a further aspect of the invention.
Also encompassed within this aspect of the invention are
functionally equivalent variants of these novel probes,
i.e. probes of equivalent specificity but of different
lengths or with modified sequences. Typically these
variant probes will be no less than 6 nucleotides
shorter, preferably no less than 3 nucleotides shorter
and no more than 10 nucleotides, preferably no more than
5 nucleotides longer than the specified probes listed

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above.   Variant probes/oligonucleotides will typically
have at least 80%, preferably at least 90%, e.g. at
least 95% sequence identity with the specific probes
listed above.

5   The probes may be designed to bind directly to the
characterising sequence, e.g. for use in FISH or for
direct hybridisation e.g. on chips using conventional
blotting techniques. The probe may be labelled or
tagged with a fluorescent marker, a radioactive marker
10   or an antigenic label.

           The sequence specific probes may identify sequences
that are specific to microbes that are associated with a
particular oil field area.

           Combinations of probes or primers may be used in
15   the method of the invention. Probe mixtures comprising
probes to identify more than one microorganism
simultaneously, or to provide a microbiological profile
therefore form a further aspect of the invention.

           The conditions under which the analysis is
20   performed may also be used to provide different levels
of specificity. For example, changing the buffer
composition in which hybridisation of the sequence
specific probe hybridises may be modified to change the
stringency. Similarly the temperature at which the
25   annealing step of the PCR occurs may be modified. Such
modifications are well known in the art.

           Also indicated within the scope of the invention
are kits for performing the methods of the invention.
Such kits comprise one or more oligonucleotides designed
30   to hybridise to or adjacent to characterising sequences
and may comprise pairs of primers for performing
amplification and/or single probes directed towards
microorganisms, these single probes may be labelled.
The selection of primers being such that a
35   microbiological profile of the sample is attained which
may be used in the evaluation of the sample for
indicators of a hydrocarbon source, for the presence of

markers of production problems, etc. In a preferred
kit, the probes are attached to a solid support, e.g. a
microchip. In this case, the probes will be designed to
hybridise to characterising sequences rather than take

5   part in an amplification reaction.

As well as oligonucleotides, the kits will
typically comprise suitable buffers for use in the test
procedure, e.g. a lysis buffer and other buffers and
components for use in an amplification reaction e.g. DNA

10   polymerase (particularly Taq DNA polymerase) and
nucleotides for incorporation into the amplified
products.

Chips to perform hybridisation analysis comprise
another aspect of the invention. The chips preferably

15   contain the probes described above. More preferably a
bacteria specific probe (e.g. SEQ ID NO: 8) plus an
archeal specific probe (e.g. with SEQ ID NO:9) plus one
or more of the probes selected from the group comprising
SEQ ID NOs: 8 to 17 or functionally equivalent variants

20   of these probes are attached. Preferably the chip
contains 2 or more, 4 or more or 6 or more
oligonucleotide probes.

The invention will now be described further with
reference to the following non-limiting Examples and the

25   figures in which:

Figure 1 shows results from agarose gel
electrophoresis of PCR products amplified with primer
sets defining Bacteria and Archaea, respectively. The
samples are reservoir 1 well 1 (1), reservoir 1 well 2

30   (2), reservoir 2 well 1 (3), reservoir 2 well 2 (4),
reservoir 2 well 2 washed interphase (5);

Figure 2 is a southern blot analysis of PCR
products from reservoir samples after DNA hybridisation
with the biotinylated probe 385SRB defining δ-

35   subdivision bacteria. The samples are reservoir 1 well
1 (1), reservoir 1 well 2 (2), reservoir 2 well 1 (3),
reservoir 2 well 2 (4);

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Figure 3 shows results from DGGE of PCR products
amplified with primer sets defining Bacteria. The
samples are reservoir 1 well 1 (1), reservoir 1 well 2
(2), reservoir 2 well 1 (3), reservoir 2 well 2 (4) and
reservoir 2, well 2 washed interphase (5);
       Figure 4 shows HaeIII RFLP types of cloned PCR
products from DNA extracted from the two reservoirs;
       Figure 5 shows RsaI RFLP types of cloned PCR
products from DNA extracted from the two reservoirs;
       Figure 6 shows differences in band migration within
clones characterised as HaeIII RFLP type A;
       Figure 7 provides DGGE results of fines received
17.8.01 amplified with primers defining Bacteria.
       Figure 8 provides DGGE results of samples from
reservoir 2 amplified with primers defining bacteria.
       Figure 9 shows the numbers of clones of various
bacterial types found in 3 wells in field 2.
       Figure 10 shows RFLP analysis of Archael samples.

Example 1

Identification of Archae and Bacteria indigenous to oil
fields.

1. Materials and Methods

1.1 Sample collection and processing

1.1.1 Sampling

Sterilized 5-L sample glass bottles (Schott) with 5 ml
of 0.1% (wt/vol) resazurin reducing agent (Sigma
Chemical Co., St. Louis, MA, USA) per bottle were
shipped to the offshore fields. Samples were collected
as raw production fluid (oil/water) from the production
flow-line before produced water separation. Bottles were
completely filled. The top oil layer sealed the water

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from the atmosphere, eliminating oxygenation of the
water phases.

5   Samples were sent onshore in sealed Al-boxes (transport
periods 1-2 weeks). During transportation the boxes were
kept at ambient temperature.

No traces of oxygen were detected in the water phases
when the samples arrived onshore.

10

1.1.2   Filtration of water samples

After arrival at the laboratory the oil and water phases
of the production fluid samples were immediately heated
15   (70°C; 20 minutes) and the hot phases separated in 2 L
separating funnels (2 L). Microbial biomass from 1 -2 L
water was collected by filtration through 0.22 μm
Sterivex GV filters (Millipore Corp., Bedford, Ma,
U.S.A) connected to Millex AP prefilters (Millipore).
20   After filtration each Sterivex filter was filled with a
2 μl lysis buffer (50 mM Tris-HCl, pH 8.0; 40 mM EDTA;
750 mM sucrose) and stored at -20°C until DNA
extraction.

25   One of the samples was highly emulsified, resulting in
only a small water phase. This emulsion phase was washed
with a sterile buffer solution, phase separated, and
filtered (approximately 1 L buffer phase) as described
above.

30

1.2 Cell counts

Cell numbers in water phases of the production fluids
were enumerated immediately after the arrival at the
35   laboratory by epifluorescence microscopy. Samples were
pre-filtered (Millex AP, Millipore), and 100 ml
filtrates centrifuged (6000 x g; 10 minutes to remove

coarse particles and residual oil droplets. The
fluorochrome 4'6-diamino-2-phenylindol (DAPI; 0.6 µg/ml)
was applied to the supernatants (10 ml), and the samples
.incubated at room temperature (10 minutes), followed by
5   filtration through 0.22 µm black polycarbonate filters
(Millipore). The filters were mounted in a fluorescence
microscope (Leitz Dialux with Ploemopak fluorescence
unit and UV-filter A and equipped with a Leica DC 100
digital camera) with immersion oil. Fluorescent cells
10   were enumerated with 1250x magnification.

1.3 Polymerase chain reaction (pcr) amplification of
reservoir samples

15   1.3.1   Nucleic acid extraction and quantification

The frozen Sterivex filters with microbial communities
were thawed and lysed directly on the filters. The lysis
was performed by incubation of each filter with 2 µg
20   lysozyme (Sigma; from a 20 mg/ml stock solution; 37° for
30 minutes). The mixtures were then incubated at 55°C
for 2 hours with 1 µg Proteinase K (Sigma; from a 20
mg/ml stock solution), and 1 % (wt/vol) sodium dodecyl
sulphate (SDS; BioRad Labs, Richmont, CA, USA) from a 20
25   % stock solution.

The lysates were transferred to sterile tubes, the
Sterivex filters washed with lysis buffer (55°C; 10
minutes), and the lysates from each filter pooled. The
30   lysates were extracted with hot
phenol-chlorophorm-isoamylalcohol (25:24:1) according to
standard procedures (Sambrook and Russel, 2001).
Briefly, each lysate (3 ml) was mixed with 6 ml hot
(60°C) Tris-HCl buffered phenol-chlorophorm-isoamyl-
35   alcohol (pH 8.0), vigorously shaken, maintained hot for
5 minutes, then cooled on ice, followed by phase
separation by centrifugation (4000 x g; 5 minutes at

4°C; Jouan Model 1812, Saint Nazaire, France). Sodium
acetate (0.2 volume of 10 M solutions) was applied to
each water phase, and these were re-extracted with 5 ml
Tris-HCl buffered phenol-chlorophorm-isoamylalcohol,

5   followed by centrifugation. The water phases were then
extracted with 5 ml hot (60°C) chlorophorm-isoamyl-
alcohol (24:1), and centrifuged as described above. The
extracted water phases were precipitated by 2.5 volumes
of 96 % ethanol (-20°C; 3 hours), the precipitates

10   pelleted by centrifugation (4000 x g), and the pellets
washed with 75 % ethanol. The pellets were dried (N_2)
after re-centrifugation and dissolved in 100 μl sterile
ultra-pure water (Biochrom AG, Berlin, Germany). The
nucleic acid extracts were frozen (-20°C).

15

Extracted nucleic acids were semi-quantified with an
ethidium bromide method (Sambrook and Russel, 2001).
Nucleic acids (2 μl) were spotted on a UV
transilluminator table (BioRad) wrapped with plastic

20   film ("GladPak"), and 2 μl ethidium bromide (10 mg/ml in
TE buffer, pH 8.0) were applied to each spot. The spots
were photographed under UV-illumination and
concentrations determined by intensity comparison to a
standard series of salmon DNA (Sigma) in the range

25   100-500 ng DNA per spot.

1.3.2   Oligonucleotides and PCR reagents

1.3.2.1   Oligonucleotides for PCR amplification

30

Oligonucleotide primers were prepared specific for
Bacteria and Archaea (Teske et al., 1996; DeLong, PNAS
USA 89(1):5685-9, 1992):

35   Bacteria
341fBac: 5'-CCT-ACG-GGA-GGC-AGC-AG-3' (forward primer
SEQ ID NO: 1)

907rBac: 5'-CCC-CGT-CAA-TTC-CTT-TGA-GTT-3' (reverse
primer SEQ ID NO: 2)
Expected PCR product: 567 bp

5   Archaea
21fARCH: 5'-TTC-CGG-TTG-ATC-CCG-CCG-GA-3' (forward
primer SEQ ID NO: 3)
958rARCH: 5'-CCC-GGC-GTT-GAA-TTC-AAT-T-3' (reverse
primer SEQ ID NO: 4)
10   Expected PCR product: 938 bp

The primers were synthesized by EuroGentec, Seraing,
Belgium. The primers were diluted in sterile water at
concentrations of 50 μM, distributed in 50 μl aliquots
15   and stored at -20°C.

1.3.2.2   Biotinylated oligonucleotides

A number of biotinylated DNA oligonucleotides (5'- and
20   3'-labelled) were prepared (Teske et.al., 1996; Massana
et. al., 1997) for Southern blotting analysis of PCR
products:

δ subdivision/gram-positive bacteria (included
25   *Desulfovibrio* and *Desulfobulbus*)
385 SRB: Biotin-5'-CGG-CGT-CGC-TGC-GTC-AGG-3'-Biotin
Hybridization temperature: 50°C (SEQ ID NO: 6)

Desulfobacter and *Desulfobacterium*
30   804 SRB: Biotin-5'-CAA-CGT-TTA-CTG-CGT-GGA-3'-Biotin
Hybrization temperature: 40°C (SEQ ID NO: 7)

Crenarchaeota (Group I *Archaea*)
554 ARCH-I:
35   Biotin-5'-TTA-GGC-CCA-ATA-ATC-MTC-CT-3'-Biotin
Hybrization temperature: 40°C (SEQ ID NO: 18)

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Euryarchaeota (Group II *Archaea*)
554 ARCH-II:
Biotin-5'-TTA-GGC-CCA-ATA-AAA-KCG-AC-3'-Biotin
Hybrization temperature: 40°C (SEQ ID NO: 19)

5

All biotinylated primers were synthesized by EuroGentec,
Seraing, Belgium. The primers were diluted in sterile
water at concentrations of 50 μM, distributed in 50 μl
aliquotes and stored at -20°C.

10

1.3.2.3 Deoxynucleotides

Stock solutions of deoxynucleotides (d'NTP) were
prepared by diluting 100 mM of the d'NTPs
15   2'-deoxyadenosine 5'-triphosphate (d'ATP),
2'-deoxythymidine 5'-triphosphate (d'TTP),
2'-deoxyguanosine 5'-triphosphate (d'GTP) and
2'-deoxycytidine 5'-triphosphate (d'CTP) (Amersham
Pharmacia Biotech, Piscataway, NJ, U.S.A). Each d'NTP
20   (100 μl) was diluted in sterile water (600 μl),
resulting in final concentrations of 10 mM of each
d'NTP. Solutions were distributed in 50 μl aliquots and
stored at -20°C.

25   1.3.3 Touchdown PCR

Extracted DNA (see above) or lysed microbial cell
suspensions were used as DNA template for PCR
amplification. When lysed cell suspensions were used
30   broth cultures were diluted in sterile water (10^{-2}) or
colonies from agar plates suspended in sterile water,
followed by heating (100°C) in 10 minutes.

A PCR mix of 100 μl mix consisted of 20 μl d'NTP (10
35   mM), 10 μl forward primer (50 μM), 10 μl reverse primer
(50 μM), 55 μl sterile water and 5 μl AmpliTaq DNA
polymerase (Perkin Elmer Roche Molecular Systems,

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Branchburg, NJ, U.S.A).

DNA template (1-10 µl) was diluted in 10 µl [10x] PCR
buffer with 15 µM MgCl$_2$ (Perkin Elmer Roche) and with
5   sterile water to a final volume of 90 µl. The mixture
was heated (95°C) in 5-10 minutes on a heating block. A
PCR mix of 10 µl was applied to each sample when the
samples were still in the heating block (95°C) and the
samples were immediately transferred to a DNA Thermal
10   Cycler (iCycler, BioRad).

PCR was run as a touchdown method to reduce the
generation of spurious by-products, and with the
following sequence cycles:
15   Denaturation: 95°C for 1 minute
Primer annealing: 65-55°C for 1 minute
DNA synthesis (primer extension): 72°C for 3 minutes
Number of cycles: 35

20   During the first 10 cycles the annealing temperature was
gradually reduced from 65 to 55°C with 1°C for each
cycle during the first 10 cycles, followed by 25 cycles
with annealing temperature of 55°C. The PCR runs were
terminated by 72°C for 15 minutes before cooling to 4°C.
25

1.3.4   Agarose gel electrophoresis

1.3.4.1   Analytical electrophoresis

30   PCR products were analysed by horizontal agarose gel
electrophoresis. Samples (27 µl) were mixed with [10x]
gel-loading TBE buffer (3 µl) (0.9 M Tris, 0.9 M borate,
20 mM EDTA, pH 8.3, 50 % (v/v) glycerol, 0.25 % (w/v)
bromophenol blue). A Low DNA Mass Ladder (Gibco BRL,
35   Paisley, UK) was used as standard, 12 µl standard in 3
µl [10x] gel-loading TBE buffer.

Gels were prepared by heating agarose (2.0 g; Sigma) in
160 ml [0.5x] TBE (0.045 M Tris, 0.045 M borate, 1 mM
EDTA, pH 8.3) in a microwave oven (4 minutes), followed
by cooling to 50°C in water bath. Ethidium bromide (10
5   μl) from a stock solution (10 mg/l ethidium bromide in
sterile water) was applied to the agarose, and the
melted gel was casted horizontally in a plastic tray
(open ends of the tray sealed) with a comb of 15-well or
20-wells in the electrophoresis apparatus (BioRad). The
10   gel was set at room temperature for 20 minutes,
submerged in [0.5x] TBE buffer, and the comb and seals
carefully removed.

Prepared samples and standard (see above) were applied
15   to the submerged gel wells (20 μl sample and 10 μl
standard) and electrophoris run with constant voltage
(150 V) for 1.5-2 hours at room temperature. Gel
documentation was performed over a UV-transilluminator
table (BioRad). The gels were photographed by
20   black-white Polaroid film (0.1 - 0.5 second exposure
time), or by digital camara (GelDoc, 2000, BioRad).

1.3.4.2   Preparative electrophoresis

25   Preparative agarose gel electrophoresis was performed
basically as described above for the analytical
approach, except that a low-melt agarose (Sigma) was
used. Agarose (1.3 g) was melted in 160 ml [0.5x] TBE
buffer or [1x] TAE buffer (0.04 M Tris-acetate, pH 8.0;
30   1 mM EDTA), the gel solution cooled to 35-50°, ethidium
bromide applied, and the horizontal gel as described
above, except that the set temperature was 4-5°C.
Samples were applied as described above, and
electrophoresis run at 100 V constant voltage for 1.5-2
35   hours.

After electrophoresis the gels were photographed and

selected DNA bands cut out from the agarose with a
sterile scalpel and transferred to microcentrifuge
tubes. Before further processing the agarose slices were
pelleted with a brief centrifugation and melted at 65°C
5   for 15 minutes. The samples were maintained melted at
35-37°C.

1.3.5   Southern blotting and hybridization

10   1.3.5.1   Blotting

By Southern blotting agarose gel electrophoresis of PCR
products were performed as described above (analytical
approach), except that no ethidium bromide was added to
15   the gel. DNA was transferred from the gel to Hybond N+
membranes (Amersham Pharmacia) by diffusion blotting.

After electrophoresis the gel was soaked in 10 volumes
of denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 2
20   x 20 minutes (slow agitation), followed by
neutralization (1 M ammonium acetate) for 2 x 15
minutes. The gel was then trimmed and placed on a glass
plate with chromatographic paper (3mm Chr, Whatman,
Maidstone, U.K.) soaked in 1 M ammonium acetate. The
25   ends of the chromatographic paper was placed into a bath
of transfer buffer (0.2 M ammonium acetate). The gel was
surrounded by a thin plastic film ("GladPak") to prevent
transfer buffer evaporation. The Hybond N+ transfer
membrane was soaked in 0.2 M ammonium acetate and placed
30   tightly on the top of the gel. Several layers of
chromatographic paper (soaked in 0.2 M ammonium acetate)
were placed on the top of the membrane, and a stack of
paper towels (5-8 cm) was placed on the top of the
chromatographic papers. A glass plate with a weight
35   (300-400 g) was placed on the top of the paper towels.

The DNA transfer was performed overnight (8-12 hours) at

room temperature, and the paper towels were changed when
they became wet. The blotting quality was controlled by
staining the gel in a bath of ethidium bromide (0.5
μg/ml in water) for 45 minutes. After blotting DNA was
fixed to the Hybond membrane under UV light for 40
seconds. The membranes were hybridized immediately or
wrapped in plastic and stored (dark) at 4°C.

1.3.5.2 Hybridization

Before hybridisation fixed membranes were prehybridised
in a solution (10 ml) of 3 x SSC, 0.1 % SDS and 1.0 %
Blocking agent (Roche Molecular Biochemicals) for 2
hours at the selected hybridisation temperature for the
different DNA probes (see above) in a Roller-Blot HB-3D
hybridiser (Techne, Cambridge, UK). Biotinylated
DNA-probes were then applied to the roller bottles in
the hybridiser and incubated at 16-20 hours at the
temperatures described for the different DNA-probes (ses
above).

After hybridisation the membranes were washed 10 minutes
in 50 ml 2 x SSC - 0.1 % SDS, 10 minutes in 50 ml 0.1 x
SSC - 0.1 % SDS, and 10 minutes with PBS-T (all washes
at room temperature). The membranes were incubated with
Extravidin-Peroxydase (Sigma Chemical Co., St. Louis,
MO), diluted 1:2000 i PBS-T for 30 minutes at room
temperature (agitation), washed 2 x 10 minutes with
PBS-T, 10 minutes with PBS (room temperature), and
developed (10-20 minutes) in 30 ml PBS with 2 tablets
diaminobenzidine (DAB; Sigma) and 24 ml 30 % water-free
H_2O_2. After development the membranes were rinsed in tap
water and photographed.

1.4 Denaturing gradient gel electrophoresis (DGGE)

1.4.1 Analytical DGGE

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By DGGE PCR products were generated with general primers defining *Bacteria* (341fBAC and 907rBAC) or *Archaea* (21fARCH and 958rARCH). To the primers 341f BAC and 21fARCH a 40 mer GC-clamp was added to the 5'-end (5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-G-3') (SEQ ID NO: 20).

DGGE was performed with 6 % (w/v) polyacrylamide (PAA) gels in [0.5x] TAE buffer (20 mM Tris-acetat, pH 7.4; 10 mM acetat; 0.5 mM EDTA) with a 20-70 % gradient of the denaturing agents urea and formamide (100 % denaturing agents corresponded to 7 M urea and 40 % (v/v) deionised formamide) in a DCode Universal Mutation Detection system (BioRad).

Stock solutions of PAA/Bis-acrylamide (Bis) (40 %) consisted of 38.93 g acrylamide and 1.07 g Bis dissolved in deionised water to 100 ml, while stock solutions of [50x] TAE buffer was generated by mixing 242 g Tris, 57.1 g acetic acid, and 100 ml 0.5 M EDTA to a total volume of 1000 ml with deionised water. Linear gradient gels (thickness 1 mm) were prepared by mixing PAA and Bis with denaturating agents to generate a 20 to 70 % linear gradient in a gradient delivery system (BioRad modell 475). Solutions with 20 % or 70 % denaturing agents are described in Table 1 below:

Table 1.
Composition of 20 % and 70 % denaturating solutions used in DGGE

CHEMICALS	Denaturating Solutions	
	20%	70%
40% acrylamide/Bis	15 ml	15 ml
[50x] TAE buffer	2 ml	2 ml
Formamide	8 ml	28 ml

Urea	8.4 g	29.4 g
Deionised water	to 100 ml	to 100 ml

For the preparation of one gel 18 ml of each solution
was mixed with 200 ml ammonium persulphate (10 % (w/v)
in deionised water) and 20 µl TEMED (BioRad), and the
mixtures immediately transferred to each of two 30-ml
syringes which were subsequently mounted in the gradient
delivery system. The gel was cast as a parallel gradient
gel (16 x 16 cm) with 1 mm thickness and allowed to
polymerize for approximately 1 hour, and with a comb of
15 wells. The electrophoresis tank was filled with [1x]
TAE buffer which was heated to 60°C in the tank, and 1-2
polymerised gels placed vertically in the
electrophoresis tank.

Each PCR product sample (10 µl) was mixed with 10 µl
sample buffer (0.05 % bromophenol blue, 0.05 % xylene
cyanol, 70 % glycerol, diluted in deionised water), and
the complete volume (20 µl) applied to each well.

Vertical electrophoresis was performed with continuous
temperature (60°C) and voltage (150 V) until both the
markers had migrated to the bottom of the gel
(approximately 4.5 hours).

After electrophoresis the gels were stained in SYBR Gold
(Molecular Probes, Leiden, The Netherlands), diluted
1:10,000 in [1x] TAE. for 20-30 minutes. The gels were
then photographed with the GelDoc system (BioRad).

The gel band patterns were compared for similarity and
similarity indices generated by the Quantity One option
of the GelDoc software program. The Dice Coefficient
method was used, based on the following formula for
similarity:

$$Similarity = 200x \cdot \frac{\sum_{l=1}^{B} Min(s_l, t_l)}{\sum_{l=1}^{B} (s_l + t_l)},$$

where S and T are vectors representing two lanes in the
same band set that are being compared.

1.4.2   Preparative DGGE

5

Preparative DGGE was performed as described for the
analytical DGGE, except that N,N'-bis-acrylylcystein
(BAC; Sigma) was used instead of Bis during gel
generation. The BAC enabled gel solution after
10   electrphoresis (Muyzer et al., 1996).

PCR samples (300 μl) were precipitated with 30 μl of 5 M
NaCl and 750 μl ethanol at -80°C for 1 hour, centrifuged
(20 000 x g, 2 minutes) in a microcentrifuge (Eppendorf
15   Model 5417C, Eppendorf, Hamburg, Germany). The pellet
was washed with 70 % ethanol, dried on a heating block
(35°C, 20 minutes) and dissolved in 30 μl sterile water.

The gel was cast, samples applied, and electrophoresis
20   run as described above.

The gel was stained after electrophoresis with SYBR Gold
(see above), and selected bands cut under
UV-illumination with sterile scalpels. Each slice of gel
25   was transferred to a microcentrifuge tube, washed 2 x 10
minutes with 100 μl sterile water, and the water
removed. β-mercaptoethanol (100 μl; BioRad) was applied
and the tubes incubated for 16-20 hours at 37°C.
Deionised water (100 μl), 0.1 volume 5 M NaCl, and 2.5

volumes ice cold ethanol was applied to each tube. The
tubes were incubated at -80°C for 2 hours, centrifuged
(10 000 x g, 20 minutes) in a microcentrifuge, and the
supernatant removed carefully. The tubes were dried
5   (35°C, 20 minutes), and the pellet was dissolved in 100
μl sterile water.

PCR product content in the samples was checked in PCR
with primers defining Bacteria, but without GC-clamp.
10 The PCR products were then purified in preparative
agarose gel electrophoresis with low-melting temperature
agarose (see above).

1.5 Cloning

15

1.5.1   TOPO TA Cloning

Cloning was performed with the TOPO TA Cloning kit
(Invitrogen, Carlsbad, CA, U.S.A), with the pCR 2.1-TOPO
20 plasmid vector and One Shot TOP10 chemically competent
Escherichia coli cells.

DNA was amplified in PCR using 341fBAC and 907rBAC
primers defining Bacteria. PCR was performed as
25 described above, except that the final termination at
72°C was prolonged to 10 minutes to generate 3'-adenine
overhangs. The PCR products were purified in preparative
agarose gel electrophoresis with low-melting temperature
agarose as described above. The gel slices with PCR
30 products were melted as described (65°C, 15 minutes) and
maintained melted at 37° until ligated into the vector.

Melted agarose slices (4 μl) were carefully mixed with 1
μl salt solution (1.2 M NaCl, 0.06 M MgCl$_2$) and 1 μl TOPO
35 pCR 2.1 vector. The ligation was performed for 10
minutes at 37°C. The tubes were placed on ice, and
transformation of the vector (4 μl) into chemically

competent TOP10 cells (50 μl) performed on ice (15
minutes), followed by heat-shock (42°C, 30 seconds), and
immediate transfer to ice (10 minutes). The
transformation reaction was diluted in 250 μl SOC medium

5   (2 % Tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM
KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose), and the
reaction in a shaking incubator at 37°C for 1 hour with
200 rpm horizontal shaking.

10   The suspensions were spread on agar plate with
Luria-Bertani (LB) agar medium (1.5 % agar, 1.0 %
Tryptone, 0.5 % yeast extract, 1.0 % NaCl, pH 7.0;
Sigma) supplemented with 50 μg/ml ampicillin or
kanamycin antibiotics. Before inoculation the plates

15   were spread with X-gal (5-bromo-4-chloro-3-indolyl
b-D-galactopyranoside; Sigma), 40 μl of 40 mg/ml X-gal
in dimethylformamide.

Suspensions (20 and 50 μl) of transformants were spread

20   on the LB agar plates (20 μl suspensions were diluted
with 20 μl SOC medium before plate spreading to ensure
even spreading). The plates were incubated for 20-24
hours at 37°C. Only transformants with inserted plasmid
grew on the medium, due to the resistance gene of the

25   plasmid. Colonies with PCR products ligated into the
vector were visualised as white colonies, as opposed to
light or dark blue colonies with vectors without PCR
product inserts. Discrete white colonies were isolated
in liquid LB medium, supplemented with ampicillin or

30   kanamycin (50 μg/ml). The medium was distributed in
24-well sterile tissue culture plates (Corning Inc.,
Corning, NY, USA), with 2 ml medium/well. The clones
were incubated 20-24 hours in the LB medium, and the
plasmids purified.

35

1.5.2   Isolation of plasmids

The plasmids were isolated with a GenElute Plasmid
Miniprep kit (Sigma), according to the instructions from
the manufacturer. Transformant clones (2 ml) were
harvested by centrifugation on a microcentrifuge
5   (Eppendorf) at 14 000 x g for 2 minutes. The pellets
were resuspended in a Resuspension Solution (200 μl) by
vortexing. A Lysis Solution (200 μl) was added, the
mixture gently inverted until clarification (8-10
times), and the lysis neutralized within 3-5 minutes
10   with 350 μl Neutralization/Binding Buffer. The cell
debris were pelleted by centrifugation (14 000 x g for
10 minutes). The supernatants were transferred into
GenElute Miniprep binding columns assembled into
microcentrifuge tubes, centrifuged (14 000 x g for 2
15   minutes), and the flow-through liquid discarded. The
binding columns were then washed with 750 μl Wash
Solution and centrifuged (14 000 x g for 2 minutes),
effluents discarded, and the columns re-centrifuged (14
000 x g for 2 minutes) to remove any additional
20   solutions. The binding columns were then transferred to
new microcentrifuge tubes, 100 μl sterile water applied,
and the tubes centrifuged (14 000 x g for 2 minutes).
The isolated plasmid solutions were stored at -20°C.

25   1.5.3   Control of positive PCR insert

Positive PCR inserts in the transformant vector was
controlled by PCR with M13 primers defining vector
sequences flanking the inserted sequence.
30
The primer sequences were:

M13 Forward primer (-20): 5'-GTA-AAA-CGA-CGG-CCA-G-3'
(SEQ ID NO: 21)
35   M13 Reverse primer: 5'-CAG-GAA-ACA-GCT-ATG-AC-3' (SEQ ID
NO: 22)

The primer sites corresponded to the bases 391-406 (M13
Forward -20) and 205-221 (M13 Reverse) of the LacZa
fragment of the vector. A plasmid without a positive PCR
product insert would result in a 202 bp M13 PCR product,
5   while a positive PCR product would result in a 769 bp
PCR product.

A PCR mix was prepared as described above (see section
3.4.3) with the M13 primer set (50 μM stock solutions).
10   Plasmid DNA template (2 μl) was diluted in PCR buffer
and sterile water to a final volume of 90 μl as
described above, mixed with PCR mix. The PCR was run
according to the following sequence cycles:
Initial denaturation: 94°C for 2 minutes
15   Denaturation: 95°C for 1 minute
Primer annealing: 55°C for 1 minute
DNA synthesis (primer extension): 72°C for 1 minute
Number of cycles: 30

20   The PCR run was terminated at 72°C (7 minutes) and
cooling at 4°C.

1.5.4   Restriction fragment length polymorphism (RFLP)

25   M13 PCR products with positive PCR product insert were
analysed with the restriction endonucleases EcoR1
(Sigma), HaeIII (Sigma) and RsaI (Sigma). The
restriction enzymes and corresponding enzymes (provided
by the manufacturer; Sigma) is described in Table 2.
30

- 50 -

Table 2   Characteristics of restriction endonucleases
and their buffers

ENZYME	A) ACTIVITY	RECOGNITION SEQUENCE	DIGESTION BUFFER	BUFFER COMP. (1 X DILUTION)
EcoRI	40 000 U/ml	5' G/AATTC 3'	Buffer SH	50 mM Tris-HCl 100 mM NaCl 100 mM MgCl2 1 mM dithioerythritol pH 7.5
HaeIII	10 000 U/ml	5' GG/CC 3'	Buffer SM	10 mM Tris-HCl 50 mM NaCl 10 mM MgCl2 1 mM dithioerythritol pH 7.5
RsaI	10 000 U/ml	5' GT/AC	Buffer SL	10 mM Tris-HCl 10 mM MgCl2 1 mM dithioerythritol pH 7.5

A) One unit of each enzyme cleaves 1 mg lDNA in 1 hour
at 37°C

Plasmid DNA amplified by M13 PCR (1, 5, or 10 μl) were
mixed with restriction enzymes: 1.0 μl EcoRI (40 U), 2.0
μl HaeIII (20 U), or 2.0 μl RsaI (20U). Each mixture was
diluted to a total volume of 50 μl with the respective
enzyme buffers [1x] concentration (see Table 2). The
reaction mixtures were incubated at 37°C for 2.5 hours
and placed on ice to stop the reaction. The enzyme
digestion was analysed as restriction fragment length
polymorphism (RFLP) on analytical agarose gel
electrophoresis.

1.6 Sequencing of plasmid DNA

For sequencing purified plasmids were PCR amplified with

the M13 primer set. DNA was measured by the ethidium
bromide method, and precipitated with 60 % ethanol and
0.1 M Na-acetate buffer (pH 4.6).

5   The precipitated PCR-products were submitted for DNA
sequencing (MedProbe).

Sequences from the plasmids were submitted to the
National Centre for Biotechnology Information (NCBI)
10   using the BLAST program of the NCBI database (Altschul
et al., 1997).

Phylogenetic trees and distance matrices were determined
with the Phylip interphase of the Ribosomal Database
15   Project (RDP; Maidak et al., 1997).

1.7 Detection of Bacteria and Archaea

The presence of Bacteria was detected by performing PCR
20   using primers 341Bac and 907rBac. Following gel
electrophoresis, the presence of a band of size 567bp
indicated the presence of bacteria in the sample (Fig.
1). The PCR products were transferred to Hybond N+
membrane by Southern blotting and subjected to
25   hybridisation with various labelled probes e.g. for the
δ subdivision bacteria including the SRB genera
Desulphovibrio and Desulfobulbus (385-SRB). Both
samples from one reservoir contained such bacteria,
whereas the samples from the other did not (Fig. 2).
30   The probe defining Desulfovibrio did not detect any of
this species in the sample (not shown).

1.8 DNA heterogeneity

35   1.8.1   DGGE

The genetic heterogeneity within the PCR products

genrated above was investigated with DGGE analysis. PCR
products defining Bacteria were concentrated 10 times
and run with a 20-70% continuous gradient of
denaturing agents. The results shown in Figure 3

5   showed that the DNA profiles of the products from the
samples varied considerably. Reservoir 1 well 1 showed
6 detected bands clustered in the lower area of the DGGE
gel, while the other sample from this reservoir showed
15 bands distributed over a large area. The two samples

10   from the 2nd reservoir showed more homogeneous results,
a total of 7-9 bands appeared in amplified DNA extracted
from the water samples or emulsion phase.

The different bands were related to define melting

15   conditions in the DGGE gel. The linear gradient
generated in the gel was in the range 20-70% denaturing
agent.

The diversity of the DGGE patterns were analysed by the

20   Dice Coefficient method.

The similarity between samples from the same reservoir
was >50%, ranging from 53.4 to 64.7% (average 58.6%).
The similarity between samples from different reservoirs

25   were <50% (range 22.2-33.5%, average 27.5%). The
differences in similarity between samples from similar
and different reservoirs were significant (P< 0.05).

1.8.2   Cloning

30

A cloning strategy was used for the differentiation of
the genetic variations in the reservoir samples.
Bacterial PCR products were inserted into the vector pCR
2.1 and transformed into competent E. coli TOP 10 cells.

35   After 3 separate clonings a number of the 101 clones
with potential Bacteria PCR product inserts were
analysed. Purified plasmids from the clones were

analysed by M13 PCR, using primers defining plasmid
sequences flanking the inserted PCR products. These
primers were selected since they were specific for
(annealed to) plasmid sequences flanking the inserted

5   PCR products. In this way possible amplification of E.
coli genomic 16S rDNA was avoided. After M13 PCR the
products were visualised in agarose gel electrophoresis.
Products with positive Bacteria PCR product inserts were
detected as a band of approximately 800 bp, while an

10   "empty" product (vector without any inserted PCR
product) was visualised as a band of approximately 240
bp (data not shown).

The results showed that most analysed clones contained a

15   positive Bacteria PCR insert (n=7/1), while a number
showed no insert (n=21) and were excluded from further
analysis. However, a few clones showed both products
(n=9). This could be the result of transformation of
more that 1 plasmid into individual clones, including

20   copies both copies of PCR product insert and of "empty"
plasmids.

Plasmids from 80 clones (clones containing PCR product
inserts) were investigated further with the restriction

25   endonucleases HaeIII and RsaI for restriction fragment
length polymorphism (RFLP)-analysis. The plasmid
sequences containing inserts were amplified (M13 PCR)
and the products subjected to RFLP-analysis. After
restriction enzyme digestion the patterns were

30   visualised using agarose gel electrophoresis.

The RFLP analysis revealed a significant variation
between the clones. No distinct differences were
observed between clones with only positive PCR product

35   inserts and clones with both positive and negative
inserts, indicating that restriction fragments were
restricted to the PCR product inserts. With HaeIII a

total of 10 different genotypes were defined (A-J), and
these are shown in Figure 4, while *Rsa*I restriction
analysis revealed a total of 8 genotypes
(a-h) as shown in Figure 5. The base pair distributions
of the digested PCR products are shown in Table 3 and 4.

Table 3
Base pair (bp) sizes of individual bands in Figure 4
(*Hae*III RFLP) as determined by comparison to the known
base sizes of the DNA standard

HaeIII RFLP type	A	B	C	D	E	F	G	H	I	J
base pairs	582	266	197	329	416	174	157	486	266	522
	89	151	122	153	204	89	130	123	228	177
	70	126	89	112	89	70	106	89	112	112
		89	70	89	70		89	70	89	89
		70		70			70		70	70

Table 4
Base pair (bp) sizes of individual bands in Figure 5
(*Rsa*I RFLP) as determined by comparison to the known
base sizes of the DNA standard

RsaI RFLP type	a	b	c	d	f	g	h	i
base pairs	676	400	405	333	251	239	196	251
	92	244	239	239	157	199	167	198
	58	92	157	111	92	157	139	141
		58	92	58	58	92	92	117
			58			58	58	58

The main genotype patterns exhibited minor or moderate
differences with respect to the mobilities of individual
bands. An example of this is shown for the *Hae*III RFLP
type A in Figure 6. Corresponding variations appeared

also for several of the other genotypes which contained
several clones.

Comparison of the HaeIII and RsaI RFLP main types showed
5 · a relationship between the patterns generated by the two
restriction enzymes. Clones exhibiting homologue
pattern with HaeIII often exhibited homologue pattern
with RsaI. A number of 22 unique types were defined
based on the combined results with the two restriction
10 enzymes, and among these 7 patterns dominated
quantitatively with ≥ 5 clones. The relationship
between RFLP patterns and reservoir origin is shown in
Table 5. These results showed that most RFLP patterns
were confined either to the one or to the other
15 reservoir, and that individual well samples from each
reservoir contained common RFLP types. Thus, the
presence of a reservoir-specific rather than a well-
specific microbial flora was indicated. A few RFLP
types also appeared in both reservoirs (Table 5).
20 However, closer examination, utilizing the high-
resolution sub-type differences, revealed reservoir
specificity also for these RFLP types, with one
exception (Table 6). A HaeIII type A-B and RsaI type
a-a appeared both in samples from reservoir 1 well 1 (1
25 clone) and reservoir 2 well 2 (2 clones).

Based on the relation between the HaeII and RsaI types a
RFLP type system was generated which took advantage of
the combined RFLP patterns generated by both restriction
30 enzymes.

Table 5

Relationship between RFLP-types and reservoir origin

RFLP type		Reservoir origin (no. clones)			
HaeIII	RsaI	1		2	
		1	2	1	2
A	a		1	1	6
A	b	2	1	2	3
A	c			1	1
A	d		1		
A	e		1	1	
B	c	1			
C	c	1			
D	b	1	1		
D	c	1			
D	g	4	1		
E	a			1	
E	c	1			
F	a	1			
G	a	3	8		
G	b	1		10	9
G	d	2		2	5
H	b	1		1	1
H	c			1	
H	e				
H	g				
I	h		1		
J	f	1			

Table 6

Comparison of RFLP-types appearing in both reservoir
samples

RFLP sub type		Reservoir origin (no. clones)			
HaeIII	RsaI	1		2	
		1	2	1	2
A-A	a-a			1	2
A-B	a-a		1		2
A-C	a-a				1
A-D	a-a				1
A-A	b-a			1	1
A-B	b-a			1	
A-B	b-c				2
A-D	b-d	1			
A-E	b-d		2		
A-A	e-c		1		
A-E	e-d			1	
H-A	b-a			3	3
H-A	b-c			1	
H-B	b-a			6	6
H-C	b-b	1			

Table 7

Further analysis of RFLP types based on the combined
patterns of the HaeII and RsaI restriction analysis.
All the clones apart from clone 13 are specific to
either reservoir 1 or 2. Reservoir 1 has oil producing
zones in the temperature range 68-75°C and reservoir 2
has oil producing zones in the temperature range 82-
90°C. It is clear that there is greater biodiversity in
the lower temperature reservoir.

RFLP type	HaeIII	RsaI	No. clones	Reservoir origin	
				1	2
1 A	A-A	a-a	3		3
1 B	A-B	a-a	3	1	2
1 C	A-C	a-a	1		1
1 D	A-D	a-a	1		1
2 A	A-A	b-a	2		2
2 B	A-A	b-c	1		1
2 C	A-B	b-a	1		1
2 D	A-B	b-c	1		1
2 E	A-D	b-d	1	1	
2 F	A-E	b-d	2	2	
3 A	A-A	c-g	1		1
3 B	A-F	c-f	1		1
4	A-A	d-a	1	1	
5 A	A-A	e-c	1	1	
5 B	A-E	e-d	1		1
6	B	c-a	1	1	
7	C	c-a	1	1	
8 A	D-B	b-c	1	1	
8 B	D-D	b-a	1	1	
9	D-A	c-b	1	1	
10 A	D-A	g	3	3	
10 B	D-B	g	1	1	
10 C	D-C	g	1	1	

11	E-A	a-a	1		1
12	E-A	c-e	1	1	
13	F-A	a-a	1	1	
14 A	G-A	a-a	5	5	
14 B	G-B	a-d	1	1	
14 C	G-B	a-f	1	1	
14 D	G-C	a-b	2	2	
14 E	G-D	a-b	1	1	
14 F	G-E	a-c	1	1	
15	G-B	b-a	1	1	
16	G-A	d-b	1	1	
17 A	H-A	b-a	6		6
17 B	H-A	b-c	1		1
17C	H-B	b-a	12		12
17 D	H-C	b-b	1	1	
18 A	H-A	c-b	1		1
18 B	H-A	c-c	4		4
18 C	H-A	c-d	1		1
18 D	H-A	c-h	1		1
19 A	H-B	e-b	1		1
19 B	H-E	e-a	1		1
20	H-B	g	1		1
21	I	h	1	1	
22	J	f	1	1	

1.9 Sequencing

A selection of clones representing unique RFLP types
were sequenced with a commercial Primer Walking Service
(MedProbe). The sequencing analysis were performed on
Bacteria PCR products generated plasmids containing 16S
rDNA PCR product inserts, with an M13 primer set which

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included plasmid regions flanking the inserted products.
The Primer Walking Service included a single strand
sequencing of both M13-amplified strands from the 5'-
starting nucleotide of each strand.

5

A number of sequences were BLAST analysed by the NCBI
database, or by the Ribosomal Database Project. For the
clones with complete sequences identified the whole 16S
rDNA product (positions 341 to 907 of the gene) was used

10   for BLAST analyses, while only parts of the product was
used for the clones with incomplete sequences. In this
way a variety of different microorganisms were
identified.

15   **Example 2**

Sample materials

Samples of "fines" were received from Statoil in several

20   batches.

The samples were of the following types: water,
water/oil, dry filtered, oil (toluene phase), emulsion
phase, washed emulsion, fibre in toluene, fibre in

25   water.

Extraction of nucleic acids
Different approaches were used for nucleic acid
extraction.

30

Phenol-chloroform-isoamylalcohol extraction
Some samples were extracted according to this method:

Samples received were immediately filtered through

35   Durapore filters (exclusion limit 0.22 μm), and the
filters were stored in 2 ml lysis buffer (50 mM
Tris-HCl, pH 8.0; 40 mM EDTA; 750 mM sucrose) at

-20° until DNA extraction.

Extraction of frozen filters were performed as follows:

5   "Fine" materials were thawed and lysed directly on
        the filters. Lysis was performed by incubation of
        each filter with 2 µg lysozyme (from a 20 mg/ml
        stock solution; 37° for 30 minutes), followed by
        incubation with 1 µg Proteinase K (from a 20 mg/ml
10   stock solution) and 1% (w/v) sodium dodecyl
        sulphate (SDS; from a 20% stock solution) at 55°C
        for 2 hours.

The lysates were treated and analysed as in Example 1.

15
Extraction with a commercial DNA extraction kit
Nucleic acids from other samples were extracted by a
simplified method using the commercial kit "Genomic Prep
DNA Isolation Kit" (Pharmacia Biotech, Uppsala,
20   Sverige).

Some samples were filtered through Durapore filters, and
the filters placed in 1 ml of "cell lysis reagent" of
the genomic prep kit. The dry filter samples were
25   crushed and incubated in 1 ml of "cell lysis reagent".
The rest of the samples were centrifuged (16000xg; 5
minutes), the supernatant discarded, and the pellet
suspended in 0.6 ml "cell lysis reagent". Suspensions
were incubated at 80°C for 10 minutes for cell lysis,
30   cooled to room temperature, 3 µl "RNase A Solution"
applied and the sample incubated at 37°C for 30 minutes.
"Protein Precipitation Solution" (200 µl) was applied,
tubes vigorously shaken (20 seconds) for protein
precipitation and centrifuged (15000xg; 5 minutes).
35   Supernatants (600 µl) were then mixed with 100%
isoproanol (600 µl), mixed, centrifuged (15000xg; 3
minutes), the pellet washed with 600 µl ethanol (70%

(v/v) and centrifuged (15000xg; 3 minutes). Pellets
were dissolved in 100 μl "DNA Hydration Solution" and
incubated at room temperature over night. Solutions of
extracted nucleic acids were stored at -20°.

5

Polymerase chain reaction (PCR) amplification
Oligonucleotides and deoxynucleotides were as described
in Example 1 and Touchdown PCR was also as described in
Example 1.

10

Analytical agarose gel electrophoresis or DGGE was then
carried out as in Example 1.

Results and discussions

15

Detection of _Bacteria_ and _Archaea_ by PCR
A selection of the extracted material was subjected to
PCR amplification by primer sets defining _Bacteria_ or
Archaea. Most of the samples described were tested in
20   PCR with both primer set.

The results of the testing could be separated in three;
a) water/emulsion phase "fines", b) oil phase "fines"
(toluene extracts), and c) results from dry filters.
25   The PCR results with bacterial primer set from the water
or emulsion phases were positive for 14 of 20 tested
samples (70%), for the oil phase 9 of 11 (82%) and for
dry filters all 7 tested samples were strongly positive.
All positive PCR products showed single DNA bands of the
30   expected size of approximately 570 bp. The PCR results
with the archaeal primers showed multiple bands of weak
to moderate intensity for of 8 of 35 tested samples.
None of these bands were of the expected size of 930 bp.
We therefore suggest that the archaeal bands were false
35   negative results, generated by some spurious primer
binding. Some of the PCR-products showed very strong
bands in agarose gel electrophoresis. These were

confined to water-samples or to samples originating from
dry filters.

The detection of positive PCR-products in the "fines"
5   recovered from the oil phase by toluene extraction has
not been reported before. It may be speculated if the
"fines" showed amphiphatic characteristics. The
bacteria may "hide" within water-containing and
protective polymeric cap that may penetrate into the oil
10   phase. In the oil phase, the outside of the "fines" may
be mainly of a hydrophobic character while the interior
is hydrophilic, capturing water and protecting the
microbes. This raises questions whether microbes may
reside and flourish within polymeric caps in oil phases
15   devoid of water. Thus, the microbes may survive simply
in water-containing polymer caps.

The PCR products with bacterial primers showed variable
results related to the individial series. Most samples
20   were extracted by a simple DNA extraction method
(commercial Genomic Prep DNA kit), showing variable PCR
results. After DNA extraction with the commercial kit
residues of oil could be visualised in several extracts,
both in water/oil and with toluene preparations, but not
25   in extracts from dry filters. However, the first series
of samples were extracted with the phenol-chloroform-
isoamylalcohol method.

With this method the PCR products showed excellent band
30   intensity in agarose gel electrophoresis, and no traces
of oil were observed in the DNA extracts. Thus the PCR-
results could be related to inhibitory oil compounds
remaining in the oil after extraction. The main reason
for not employing the phenol-chloroform-isoamylalkohol
35   method on all samples was the labour intensity of this
method. The results therefore emphasised the
requirements for optimising DNA extraction procedures.

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A modified phenol-chloroform-isoamylalkohol method will enable the extraction of larger numbers of samples.

In conclusion bacteria were detected in samples
5. recovered both from most samples tested, including both water/emulsions/oil phases, oil-phases (recovered in toluene), and dry filters. Of special interest was the detection of bacterial DNA in the oil-phase samples. The culture-independent methods used here enabled
10 microbial analysis despite destructive sample pre-treatment, which resulted in killing of the microbes present in the samples.

DGGE analysis
15 DGGE analyses of a selection of the samples were performed with PCR products amplified with bacterial primer set. The results are visualised in Figure 7 and summarised in Table 7.

20 **Table 8**   A summary of the DGGE results described in Figure 7.

	Samples	Type of Sample	Received Dates	Bands (no.)	A) Band migration (relative front)
25	90B	water	14.08.01	(1)	73
	A-36	water	14.08.01	1	74
	A-14	water/oil	14.08.01	6	37,52*,64,70,78*
	A-13	water/oil	14.08.01	6	34,45,52*,73,77*
	A-48	water	14.08.01	2	50, 73
30	A-09B	water (not filtered)	14.08.01	2	27, 38, 51*
	A-14	water (not filtered)	14.08.01	3	26, 36, 49*
	HD-A-09B	Dry filter	14.08.01	3	26, 36, 49*
	1	oil (toluene phase)	28.09.01	1	69
	2	oil (toluene phase)	28.09.01	2	48, 69
35	3	oil (toluene phase)	28.09.01	1	69
	4	oil (toluene phase)	28.09.01	0	---
	5	oil (toluene phase)	28.09.01	1	66

6	oil (toluene phase)	28.09.01	1	69
7	oil (toluene phase)	28.09.01	1	69
A-52	emulsion phase	28.09.01	1	69
A-09B	emulsion	28.09.01	(1)	66
A-09	washed emulsion	28.09.01	1	68
A-36	emulsion	no information	4	41,54,64*,66*, 68*
1	dryfilter	23.10.01	2	49,72
2	dryfilter	23.10.01	2	49,71
3	dryfilter	23.10.01	4	49, 56, 63*, 72*
4	dryfilter	23.10.01	5	48,56,63,66*,71
5	dryfilter	23.10.01	6	27, 29, 51, 56, 61*,64*, 71*
6	dryfilter	23.10.01	2	48,70
A-36 (1)	oil/emulsion/water	14.11.01	2	48,78
A-36 (2)	oil/emulsion/water	14.11.01	2	48,71
A-09B (3)	oil/emulsion/water	14.11.01	2	49, 70

A) The numbers marked with asterisk were those visually considered to be the main DGGE bands by intensity

Example 3

Correlation of bacterial species present with different wells in field 2.

The methods described in Examples 1 and 2 were used to study the diversity of microbial populations in 3 different wells in field 2.

Figure 8 shows the DGGE bands seen when DGGE was performed on PCR products generated using the bacteria specific probes outlined above. The fact that different DGGE banding is seen for the three wells indicates that the microbiological profile overall may be different. This was investigated further by cloning and sequencing the PCR products as described in Examples 1 and 2. The pattern seen with simply performing DGGE can give an indication of the microorganisms present (for example A14 gives a typical pattern of Arcobacter).

        The results of the cloning and sequencing are shown
in Figure 9, which shows the number of clones of the
various species present in the three wells. Only α
proteobacteria are found in more than one well.

5   This shows that mapping of a sample to an
individual sample well, within a reservoir is possible.
This may also be linked further to particular reservoir
zones or with a particular mineralogical oil type. The
well may also be surveyed with respect to its production

10 and/or the data could be linked to the temperature range
of the particular reservoir zone.

Example 4

        The RFLP type of Archaea was investigated in

15 samples taken from three different North-Sea seeps. The
methods were carried out as above and the results
further substantiate the fact that different
microbiological profiles can be correlated with samples
being taken from different locations. For example RFLP

20 type I is found at all 3 locations whereas RFLP types 3,
6 and 7 are each found at only one location (Figure 10).